TLR-9 Expression in Human Bronchial Epithelial Cells induced with Lipopolysaccharide

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Abstract

The study conducted here was to investigate the gene and protein expression of Toll like receptor 9 when human bronchial epithelial cells were induced by lipopolysaccharide. TLRs are pattern recognition receptor (PRR) which plays a key role in innate immunity. They recognise the molecules that are shared by pathogens but distinguishes from the host which is referred to as pathogen associated molecular patterns (PAMPs). The HBECs were first cultured and once it becomes confluent they were induced with 10ng/ml of LPS. In order to find out whether the gene was expressed in the cells, the RNA was isolated and RT-PCR was carried out. Before carrying out the RT-PCR, RNA gel electrophoresis was carried out to show the 28S and 18S bands. Once the gene expression was analysed protein expression was carried out by extracting the protein using RIPA buffer and running the SDS-PAGE followed by staining. Silver staining and western blotting results revealed clear bands at 116kDa illustrating that the TLR9 protein had been expressed. RT-PCR results showed that a smear of bands for TLR9 and the in protein expression bands were seen in TLR9 protein region which indicates that TLR9 was expressed in the HBEC by LPS.

Keywords: *Toll like receptor, PAMPs, LPS, HBECs, RIPA*

1. Introduction

The aim of the study was to know whether Toll like receptor 9 (TLR9) is expressed when the human bronchial epithelial cells were induced with Lipopolysaccharide. The gene and protein expression analysis were made in this study.

1.1 An brief introduction on the immunity and immune response

Immunity refers to all the mechanism involved in the organism for the protection of body against pathogens such as bacteria, fungi, viruses, and wide range of foreign substances. Any foreign substance that is capable of inducing the immune response and in the production of antibodies is called as immunogens. Foreign substances, microbes or foreign particles that are capable of interacting with the immune system products are called as antigens. All antigens have the ability to bind to its respective antibody but they may not have the immunogenic capacity (ability to induce immune response). There are various types of immunity of which overview of two basic types are discussed namely, acquired and innate immunity. The immunity due to the exposure of various antigens is called acquired immunity which will produce immunological memory (*Hunt* 2007).These are non-specific immunity which is the first line defence system and doesn't produce immunological memory (*Hunt* 2007).

1.2 TLR – Toll like receptors

1.2.1 What are TLR's?

Toll like receptors are type I transmembrane proteins characterized by an extracellular leucine-rich domain and a cytoplasmic domain. Since they have a homolog with the cytoplasmic domain of the Interleukin1 receptor (IL-1R) they are referred to as Toll/IL-1R domain or TIR domain. They belong to pattern recognition receptor (PRR) which plays a key role in innate immunity. They recognise the molecules that are shared by pathogens but distinguishes from the host which is referred to as pathogen associated molecular patterns (PAMPs).

1.2.2 Structure of TLRs

The common structure for the mammalian TLR family has the following features: multiple leucine-rich repeats and one or two cysteine-rich regions in the large and different ligand-binding ectodomain; a short transmembrane region; and has a highly homologous conserved cytoplasmic domain among the individual TLRs. As the cytoplasmic domain of the interleukin 1 receptor (IL-1R) TLRs also possess a similar one Toll/IL-1R (TIR) domain (*Laurent et al* 2005).

1.3 Different types of TLR's and their respective ligands

1.3.1 TLRs Types

These belong to the TLRs family and identify pathogen associated molecular pattern which is specific for gram-positive bacteria. TLRs encode a protein which recognizes the pathogen and activates the innate immunity. Both the human and drosophila TLR are highly conserved and shares the structural and functional similarities. TLR 1 mediate the production of cytokines by

recognizing the pathogen associated molecular patterns (PAMPs) expressed in the pathogen. There are thirteen classes of human TLRs have been found so far identified TLR (1-13).

1.3.2 Ligands of TLRs

The TLR family members are used as critical signal transducers by some pathogen-associated molecular patterns and host-derived products. The LPS from Gram-negative bacteria is essential for TLR 4 signalling; the LPS from *Leptospira* and *P. gingivalis* are recognized by the TLR 2 and not by TLR 4. The endogenous host-derived products, such as hsp60 and fragments from fibronectin are also identified by TLR 4. TLR 5 recognizes flagellin and TLR 9 CpG DNA for signalling. (*Akira et al*., 2001).

1.4 Role of TLR in innate immunity

TLRs can induce the production of different types of cytokines such as IL-12 and IL-18 in antigen presenting cells (APCs) when they recognize the pathogens or their products. The cytokines that are produced will function as a useful cytokines to force naive T cells to differentiate into T_H1 cells. Pathogens are usually captured in multiple ways like phagocytosis, endocytosis or *via* TLRs themselves and the pathogens captured are then processed & presented to T cells as major histocompatibility complex−antigen (*Ito et al* 2002). Antigen presentation requires connected up-regulated expression of co stimulatory molecules on the cell surface of APCs for expansion of antigen-specific T cell clones, which is also triggered by TLR signaling. The role TLR-stimulated APCs do is mainly inducing T_H1 development and it still remains unclear whether they are involved in T_H2 development. (*Akira et al.,* 2001).

1.5 Signalling pathway of TLR

TLRs facilitates dimerization with other TLRs when it recognises the microbes i.e., TLR2 is shown to form heterophilic dimer with TLR1 and TLR6 in some case they are believed to form homodimers. The dimerization activates the signalling pathways which originate from the cytoplasmic TIR domain. For the induction of inflammatory cytokines such as TNF-α & IL-12 through all TLRs MyD88 (myeloid differentiation primary-response protein 88- a TIR domain containing adaptor) is essential. The gene expression use to be slightly different for specific TLR activation, which indicates that signaling pathway is divergent although MyD88 is common implies there are MyD88-dependent and MyD88-independent pathways (*Muzio et al.,* 2000). When TLRs are triggered it associates with the MyD88, allowing the association of IRAK1 (IL-1R-associated kinase 1) by recruiting IRAK4. Phosphorylation of IRAK1 is then induced by IRAK4, phosphorylated IRAK1 associates with the TRAF6 (tumour-necrosis-factor-receptor-associated factor 6). Phosphorylated IRAK1 and TRAF6 forms a complex with TAK1 (transforming-growth-factor-β-activated kinase), TAB1 (TAK1-binding protein 1) and TAB2 at the plasma membrane inducing the phosphorylation of TAB2 and TAK1 (*Akira & Takeda* 2004). A complex consisting of TRAF6, TAK1, TAB1 and TAB2 translocates to the cytosol, when IRAK1 is degrades at the plasma membrane. The complex then associates with ubiquitin ligases UBC13 (ubiquitin-conjugating enzyme 13) and UEV1A (ubiquitin-conjugating enzyme E2 variant 1), leading to the ubiquitylation of TRAF6. TAK1 gets activated by the ubiquilated TRAF6, activated TAK1 phosphorylates both mitogen-activated protein (MAP) kinases and the

IKK complex (inhibitor of nuclear factor-κB (IκB)-kinase complex), which consists of IKK-α, IKK-β and IKK-γ (also called as IKK1, IKK2 and nuclear factor-κB (NF-κB) essential modulator, NEMO, respectively). The translocation of NF-κB to nucleus for the induction of the specific gene occurs when IKK complex phosphorylates IκB leading to the ubiquitylation and subsequent degradation (*Akira et al.,* 2004).

1.6 MyD88 Dependent pathway

Study on MyD88-deficient cells shown the existence of MyD88-dependent and -independent pathways, both of which intervene signalling in response to LPS. For example for the TLR2 (mycoplasmal lipopeptide) ligand the activation of NF-κB is completely abolished when MyD88-deficient macrophages, in case of TLR4 (LPS) ligand the response is observed with delayed kinetics. It's also noted that MAPK activation is delayed in LPS-stimulated MyD88-deficient macrophages (*Akira & Takeda*; 2004).

1.7 MyD88 Independent Pathway

The (IFN)-regulatory factor (IRF3) will be activated in MyD88-independent pathway activates which will lead to the production of IFN-ß and the expression of IFN-inducible genes. According to the MYD88 independent pathway the TLR molecules activates the Interferon Regulatory Factor (IRF-3) that in hand induces the interferon – β synthesis. The interferon molecule produced in the cytoplasm reaches the extracellular matrix and self activates the other co-stimulatory genes for interferon production in the cell through stat 1 molecule. It also involve in the late phase and leads IFN-ß production & IFN-inducible genes expression (*Akira & Takeda* 2004).

1.8 Role of TLR9 in triggering innate and adaptive immunity

Irrespective of presence or absence of CpG motifs, DNA is endocytosed into the cellular compartment where it is exposed to TLR9, which is activated if the DNA contains CpG motifs. Activation of TLR9, in pDC is dependent on interleukin (IL)-1 receptor-associated kinase (IRAK)-4 and interferon regulatory factor-7(IRF7), and requires direct interactions between IRF7 and MyD88, tumour-necrosis factor-α(TNF- α) receptor activated factor-6 (TRAF6) and IRAK-1. Activation of TLR9 results in induction of nuclear factor-κB(NF-κB) and other intracellular signalling pathways that trigger a rapid innate immune response which is characterized by the secretion of a variety of proinflammatory and antiviral cytokines, such as IL-6, TNFα and type 1 interferon's(IFN), and immune regulatory cytokines that limit the intensity of the inflammatory response, such as IL-10. There is also a converse effect of CpG-activated B cells on dendritic cells, by which TLR9 activation drives CD5 B cells to produce IL-10, which then suppresses the T_H1-priming function of the dendritic cells. In contrast to some other innate immune responses, relatively little IL-12 is produced by TLR9-activated human immune cells. Secondary activation of Natural killer (NK) cells and other innate immune cells is done by pDC through both IFN-dependent and independent pathways. B cells activated through TLR9 have a greatly increased sensitivity to antigen stimulation, promoting their differentiation to antibody-secreting plasma cells, and therefore contributing to the adaptive immune response (Marshall *et al.,* 2003). TLR9 stimulated pDC migrate to the T-Cell zones of lymph nodes and other secondary lymphoid tissues where it express increased levels of co-stimulatory molecules

that enhance their capacity to activate naïve and memory T cells, and have increased capacity to cross-present soluble protein antigens to CD8 T cells. As a result, CpG DNA promotes strong TH1 CD4 and CD8 T-cell responses (*Kreig et al 2006*).

2. Materials and Methods

2.1 Cell culture

Cells were cultured using DMEM media which was obtained from the invitrogen^{TM} and the media were altered by mixing 45ml of fetal bovine serum (FBS), 5ml of penicillin-Streptomycin (1%) to the 500ml DMEM media. Human bronchial epithelial cells (HBEC) were cultured in the T75 flask with the media prepared. The cells were kept in the incubator so as to allow the growth and avoid the contamination. The cells were supplied with fresh media for a period of 24 hours to allow them grow faster. Once the cells became confluent they were removed from the incubator for the process called trypsinization, which was carried out to dissociate the cells in the container for the purpose of splitting the confluent cells in the flask into two. The step includes the addition of 5 ml trypsin with 45 ml of 1 times PBS to digest the proteins that make the cells adhere to the container. One important step in this method is that, when the cells started to float the media should be added to the flask to avoid the action of trypsin on the cells. Then the cells were induced with the 5ng/ml of LPS for the first time and 10ng/ml of LPS in the second time. The concentration was changed in the second time since there was no expression observed at 5ng/ml.

2.2 Gene expression

2.2.1 RNA Extraction

The confluent cells were lysed by adding RNA STAT-60^{TM} (5ml) by passing the cell lysate several times into the flask. Before the addition of the RNA STAT-60^{TM} to the cells it should not be washed because of the chance of mRNA degradation. After the process of homogenization the homogenate was stored at room temperature so as to permit the complete dissociation of the nucleoprotein complexes. 1ml of chloroform added to the RNA STAT-60^{TM}, covered tightly, shaken vigorously for 15 to 30 seconds and left at room temperature for 2-3minutes. The homogenate was centrifuged at 12,000g to the max for 15 minutes at 4^0C. Centrifugation separated the homogenate into two layers red lower phenol chloroform phase (lower phase) and the colourless upper aqueous phase. RNA remains in the aqueous phase whereas the DNA and proteins was in the interface and organic phase. The volume of the aqueous phase was about 60-65% of the volume of the RNA STAT-60^{TM} that was used for homogenization. The upper aqueous phase was transferred to a fresh tube and mixed with isopropanol (2.5ml). Samples were stored at room temperature for 5-10minutes and centrifuged for 12,000g for 10 minutes at 4^0C. White pellet formed at the bottom of the tube is the RNA precipitate which is often visible before centrifugation. Supernatant was removed and the RNA pellet washed once with 75% ethanol by vortexing and subsequent centrifugation at 7,500g for 5minutes at 4^0C. The RNA pellet was dried by air-drying or in a vacuum (5-10 min) and it was made sure that it doesn't get dried up completely which might decrease the RNA solubility. RNA pellet was dissolved in 1 mm EDTA vortexed and then the pellet was pipette few times. In order for the RNA samples to dissolve they

were incubated at 55-60^{0}C, for the process of solubilisation RNase-free solutions treated with Diethylpyrocarbonate (DEPC) were used in RNA.

2.2.2 Gel Electrophoresis of RNA

1.1% agarose gel was prepared in running buffer. In a microfuge the alcohol precipitates of nucleic acids were centrifuged for five minutes. The tubes were drained thoroughly and completely dissolved in the pellet to a concentration of 2 µg/4µl in 10 mM phosphate buffer. Two 4 µl aliquots were transferred to polypropylene tubes. One of the tubes was added with 8 µl of phosphate buffer and kept on ice. To the other 8 µl GFP mix was added and the tubes were vortexed and incubated at 55°C for 15 minutes. To each sample 3 µl of loading mix was added, thoroughly mixed, and then loaded with 8 µl on the gel (Barbero *et al.,* 2006).

2.2.3 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The process of synthesis of DNA from an RNA template by means of a reverse transcriptase commonly referred to as RT-PCR where RT refers reverse transcription.
Primers for human TLR9
Forward primer **GGACCTCTGGTACTGCTTCCA**
Reverse Primer **AAGCTCGTTGTACACCCAGTCT** (Zarember & Godowski 2002)
First Strand cDNA synthesis
Contents taken in PCR tube pipette is given in table 1

The tubes were vortexed gently for 10 seconds to mix the components in the tube and placed in a controlled temperature heat block at 48^{0}C & incubated for 45minutes. To inactivate the AMV reverse transcriptase and to denature the RNA/cDNA/primer it was heated at 94^{0}C for 2minutes.

Second Strand cDNA synthesis and PCR amplification

The PCR tubes were placed in the PCR system and the PCR program was started as mentioned in table 2. 2% agarose gel was prepared and in a separate eppendorf tube 1 µl of the PCR mixture added with 1 µl of mini gel loading buffer and mixed. The PCR products were loaded to the agarose gel and in one lane the DNA molecular weight markers were added and the yield of the PCR product estimated.

2.3 Protein Expression

2.3.1 Protein Lysis

RIPA (Radio Immuno Precipitation Buffer) buffer was used for the lysis of the cells from the cultured flasks. First the culture medium from the adherent cells in the flask and the cells were washed twice with cold PBS (X 1). Cold RIPA buffer was added to the cells and 1ml of the buffer and kept on ice for 5 minutes. The plates were swirled for equal spreading of the buffer; the cell lysate was collected towards one end of the flask using the cell scraper. Cell lysate was transferred to a micro centrifuge tube and centrifuged at ~14,000 X g for 15 minutes to pellet the

cell debris. In order to increase the yield the pellets were sonicated for 30 seconds with 50% pulse, the supernatant was collected to a new tube for the further process.

2.3.2 SDS-PAGE Gel Electrophoresis

Materials

Acrylamide 30% and Bis acrylamide 0.8% was prepared by mixing 75.0 gram of acrylamide and 2.0 g of Bis acrylamide and made up to 250 ml using water. Separating Gel Buffer requires 1.875 M Tris-HCl buffer with pH 8.8 was prepared by mixing 113.5 g of Tris-HCl buffer in 500 ml of water and the pH adjusted to 8.8 with HCl. Stacking Gel Buffer includes 0.6 M Tris-HCl buffer with pH 6.8 was prepared by mixing 36.6 g of Tris –HCl buffer in 500 ml water and the pH adjusted to 6.8 using HCl. Electrode Buffer was made with Tris of 0.05 M (12 g), Glycine of 0.384 M (5.76 g) and SDS of 0.1% (2 g) was made up to 2 litres using water without any pH adjustments. To 5.0 ml of the stacking gel buffer, 0.5 g of SDS, 5.0 g of Sucrose, 0.25 ml of Mercaptoethanol and 5.0 ml of Bromophenol Blue of 0.5% is made up to 50 ml using water thus the sample buffer was prepared. Ammonium per sulphate 10% (APS) was freshly prepared and 1 g in 10ml is used. TEMED (N.N.N`.N`.Tetra methyl ethylene diamine) was provided and 0.1% Coomassie Brilliant Blue R250 was mixed in 50% methanol first and then10% glacial acetic acid was added to the above mixture for staining purpose. 10% methanol and 7% glacial acetic acid was used as a destain solution.

Method

Samples to be run were denatured in the sample buffer by heating for two minutes at a temperature range of 95-100°C. Cell homogenates which has to be run was prepared at a final concentration of 5-10 mg/ml. The plates were joined to form the cassette after cleaning them using methylated spirit and drying. Separating gel was prepared by mixing the following components in the same order and quantity the separating gel was prepared. The mixture was properly degassed as oxygen will intermit the polymerization of acrylamide and bis acrylamide. Table 3 shows the composition of chemicals requires to make a 10 and 15% gels. The gels prepared were 7.5% and the half of the 15% gel was taken at a half amount for a litre of solution. After degassing 5 µl of TEMED was added to the mixture which enables the polymerization reaction. Using 1ml Gilson, the separating gel was transferred in to the gel cassette carefully till it reaches 0.3 cm from the bottom of the comb. To ensure that the gel sets with a smooth surface, distilled water was poured down one edge between the glass plates until a layer of about 2mm exists on top of the gel solution and then allowed the gel to get set. Changes in refractive index were observed in between the water and the gel. After the gel gets set, water over the separating gel was removed with Whatman No. 1 chromatography paper folded over to provide a double thickness. The stacking gel was prepared by mixing the following components in the same order and quantity the stacking gel was prepared. The mixture was properly degassed as oxygen could intermit the polymerization of acrylamide and bis acrylamide, the stacking gel composition was taken as shown in table 4. After degassing the stacking gel mixture 5 µl of TEMED was added and the contents were discharged in to the plates carefully till it reaches the cut away edge of the gel plate. Comb of specific size was placed in to the solution of stacking gel in between the plates for the well formation. The comb and the spacer below the gel was carefully removed

from the gel cast and the gel cassette was placed in the electrophoretic tank. Top reservoir was filled with the buffer till it floods over and fills the wells. 300 ml of buffer was added to the bottom section cautiously; approximately 5 μl of unknown sample and the standards were loaded in to the wells slowly. Finally the apparatus was connected to the power pack that produced a constant voltage of about 200V. After the gel has completely run, the gel is removed carefully from the cassette and then staining was carried out overnight in a shaker. Once the staining was over the destaining was done and the bands were observed under transilluminator.

2.3.3 Staining and De-staining

2.3.3.1 Coomassie Staining

The most common staining method for the SDS-PAGE coomassie blue stain was used to view the protein bands in the gel. To 50% ,ethanol 0.1% of coomassie brilliant blue R250 was dissolved and 10% glacial acetic acid added to the solution. The solution was stirred in a magnetic stirrer until the components are mixed thoroughly. After mixing the staining solution was added to the gel and for every half an hour the gel was washed with water and the solution was replaced. This was followed 3-4 times and left for the overnight in shaker; once the staining step was completed destaining was done. In destaining 10% methanol was mixed with 7% acetic acid and the solution was added to the stained gel and left for two days. After two days of time the gels were viewed in UV light and the bands were photographed for analysis.

2.3.3.2 Silver Staining

The bands obtained in the coomassie blue stain was not so clear hence silver staining was carried which is more sensitive than the coomassie blue staining and accurate. The gel was stained with silver to obtain clear view of the proteins. Firstly the gel was fixed for 30 minutes in 10% glutaraldehyde of 100 cm^3 and then the gel was rinsed with distilled water for overnight. Every time the water used was changed to obtain good results. After overnight washing the gel was soaked in 5 g cm^3 of dithiothreitol for 30 minutes. The solution was removed and 0.1% silver nitrate was added without rinsing and kept for 30 minutes. After sufficient staining was reached the staining was stopped by washing with water.

2.3.3.3 Western Blotting

Western blot is one of the widely used methods for the analysis of the protein expression we opted for the use of western blot analysis as a confirmatory test for this protein expression study. In general it is method which isolates one protein from a mixture of proteins by means of a unique high quality antibody directed towards a particular protein. The method usually involves four major steps which are as follows:

i) The proteins that were obtained from the gel electrophoresis were taken.
ii) With the use of nitrocellulose paper the gel containing the separated proteins were transferred on to it.
iii) The primary antibody purchased was added to the nitrocellulose paper.
iv) Secondary antibody was used to make an antibody-enzyme conjugate (Sakudo, 2006; Westermeier, 2005).

3. Results

3.1 Gene Expression

3.1.1 Gel Electrophoresis of RNA

In order to evaluate the RNA purity and quality, RNA gel electrophoresis was performed (Fig 1). Typical 18S and 28S bands were observed in the electrophoretic pattern showed the typical 18S and 28S bands which indicate that there was no degradation and no genomic DNA contamination. After which the RNA samples were subjected to RT-PCR.

3.1.2 RT-PCR

The RNA samples where amplified as given in the methods and the amplified samples were collected from the PCR. Gel was run to know whether the amplified product contains TLR9 in it so that it can be confirmed that the gene has been expressed in the cells (Fig 2). In the lanes hunt for two TLR's was done TLR4 and TLR9 and the end of both the lanes the 1Kb markers were loaded.

3.2 Protein Expression

In order to show that protein was expressed the cells induced by LPS was collected and the proteins were extracted as mentioned in the methods. SDS-PAGE was carried out to analyze the protein sample.

3.2.1 SDS-PAGE

The SDS-PAGE gels were run at 200V for 40 minutes and once when the samples moves towards the other end of the gel they were removed out of the apparatus and stained to view the proteins in that.

3.2.1.1 Coomassie blue staining

Staining was done with coomassie blue first to see the proteins in the gel, once the gel was stained it was kept for an overnight and destained. (Fig 3)

3.2.1.2 Silver Staining

Since the bands in the coomassie blue stain was not so clear the silver staining was carried out in which the bands where very clear showing the respective proteins. (Fig 4)

3.2.1.3 Western Blotting

Both the staining tests cannot be taken as a confirmative test western blotting was carried and the results of the western blotting showed the clear presence of TLR-9 indicating the induction by LPS. (Fig 5)

4. Discussion

The members of TLR family are expressed on immune cells and play different roles in PAMP signalling. Engagement of the TLRs by their respective ligands triggers a multifaceted response involving antigen presentation, expression of co stimulatory molecules, and release of cytokines, which in turn stimulates adaptive responses involving T and B-lymphocytes. HBEC's when induced with the Lipopolysaccharide showed the gene and protein expression of TLR-9.

4.1 Gene Expression

4.1.1 RNA Analysis

In the gene expression the RNA was first extracted and RNA gel electrophoresis was carried out for the 18S and 28S RNA. From the gel electrophoresis results it was clear that there was no RNA degradation or any genomic DNA contamination in the sample (Figure 1). Then the cDNA was prepared from the RNA samples and amplified using the PCR and the results of the PCR showed that the gene was expressed. The result also suggest allows to know the mRNA quality since mRNA comprises only 1-3% of total RNA samples and it is not readily detectable even with the most sensitive of methods. On the other hand Ribosomal RNA comprises >80% of total RNA samples, with the majority of that comprised by the 28S and 18S rRNA species (in mammalian systems). Hence the mRNA quality was assessed by electrophoresis of total RNA followed by staining with ethidium bromide (*Palmer & Prediger,* 2008).

4.1.2 Gene expression studies from PCR results

From the PCR results TLR9 was found to be expressed at lower levels (trace amount of bands seen in Fig 2), at the same time the amount of other TLR expression was very less compared to TLR9. Expression of TLR9 indicates that it has more response compared to the other TLR's. Apart from TLR9, TLR4 response had also been observed at a lower level from the PCR results (Figure 2). Clear bands in the region of 312kb indicate that the gene expressed was TLR9 and the bands where clearly visible in different kinds of dilutions which were in different lanes. Though all the dilution shows a smear of bands the results clearly indicates that the gene was expressed hence so to confirm the gene expression the protein expression was also carried out to support the report. Previous studies on mice showed that the stimulation with LPS could up regulate gene expression of TLR9, indicating that cells with increased TLR9 expression induced by LPS might respond to invading bacteria more effectively (*An et al*., 2002). These results indicate that the TLR9 expression has a role with the LPS and from the results shown in fig 2 it is very clear that the gene has been expressed by the HBECs when induced with LPS. One other important thing to be noted in this experiment is that the TLR were expressed at a high concentration of LPS i.e., 10ng/ml. At first when 3 and 5ng/ml of LPS were tried the cells dint express the TLR's which indicates that the level of LPS required for the induction of TLR's may be 10ng/ml. A previous

study also indicates that the amount of LPS used for the production of TLR response were around 10ng/ml (*Jones et al., 2000*).

4.2 Protein Expression

4.2.1 SDS-PAGE

Though the results were observed to be a trace in gene expression it was clearly visible that gene has been expressed to add more support to this statement protein expression was carried out. In the protein expression the proteins were isolated from the cells that were induced by the LPS, isolated and run in the SDS-PAGE. Then they were stained with the coomassie blue stain and the results showed a band at 116kda in the coomassie blue staining (Figure 3). The band that was obtained in the coomassie blue staining was not clearly visible so the gel was stained with silver staining. Silver staining is an accurate and sensitive method compared to the coomassie blue staining. Hence so the results showed lots of clear bands that were not visible in the coomassie blue staining. The bands for TLR9 at 116kda were clearly visible in this staining in two different concentrations. The protein expression showed bands that were necessary to show that the gene expressed was TLR9 (Figure 4). The western blot results confirms the expression of TLR-9 in the HBEC by LPS, which can be shown through the clear bands at 116kDa indicating its presence. Finally though the results may show smear of bands in the RT-PCR and silver staining, it can be confirmed that the gene or the protein for the TLR9 has been expressed in the cells with the results of western blot analysis. Since TLR9 recognize CpG motifs (*Akira et al*., 2001) which was not added to the cells it can be stated that LPS can induce the TLR-9 expression and also studies showed that LPS up regulates the TLR9 expression (*An et al*., 2002). It was shown in a study that expression of TLRs in non immune cells may cause autoimmune inflammatory disease (*Kohn et al.,* 2005).

5. Conclusion

Thus it can be concluded that the TLR9 can be expressed by lipopolysaccharide which may provide a novel approach to the researchers in innate immunity. Studies are being carried out in finding out more about the up regulation of the TLR9 expression. It can also be extended in finding out the expression of TLRs in non immune cells which results in the expression of an autoimmune inflammatory disease for example Hashimoto's thyroiditis (TLR3 in thyrocytes), colitis (TLR4 in intestinal epithelial cells).

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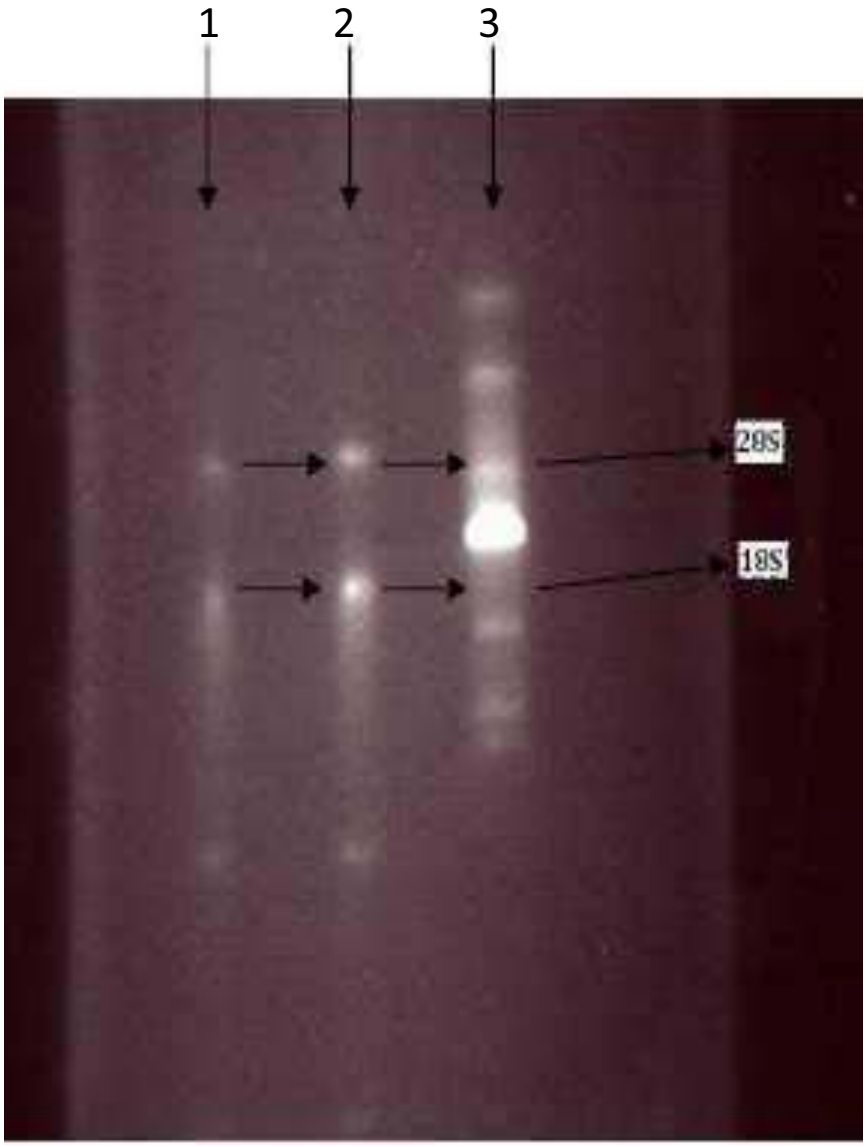

Figure 1 Showing the Gel Electrophoresis of RNA from the HBECs induced with LPS

1, 2- RNA Sample showing the 18S and 28S 3-Molecular weight marker

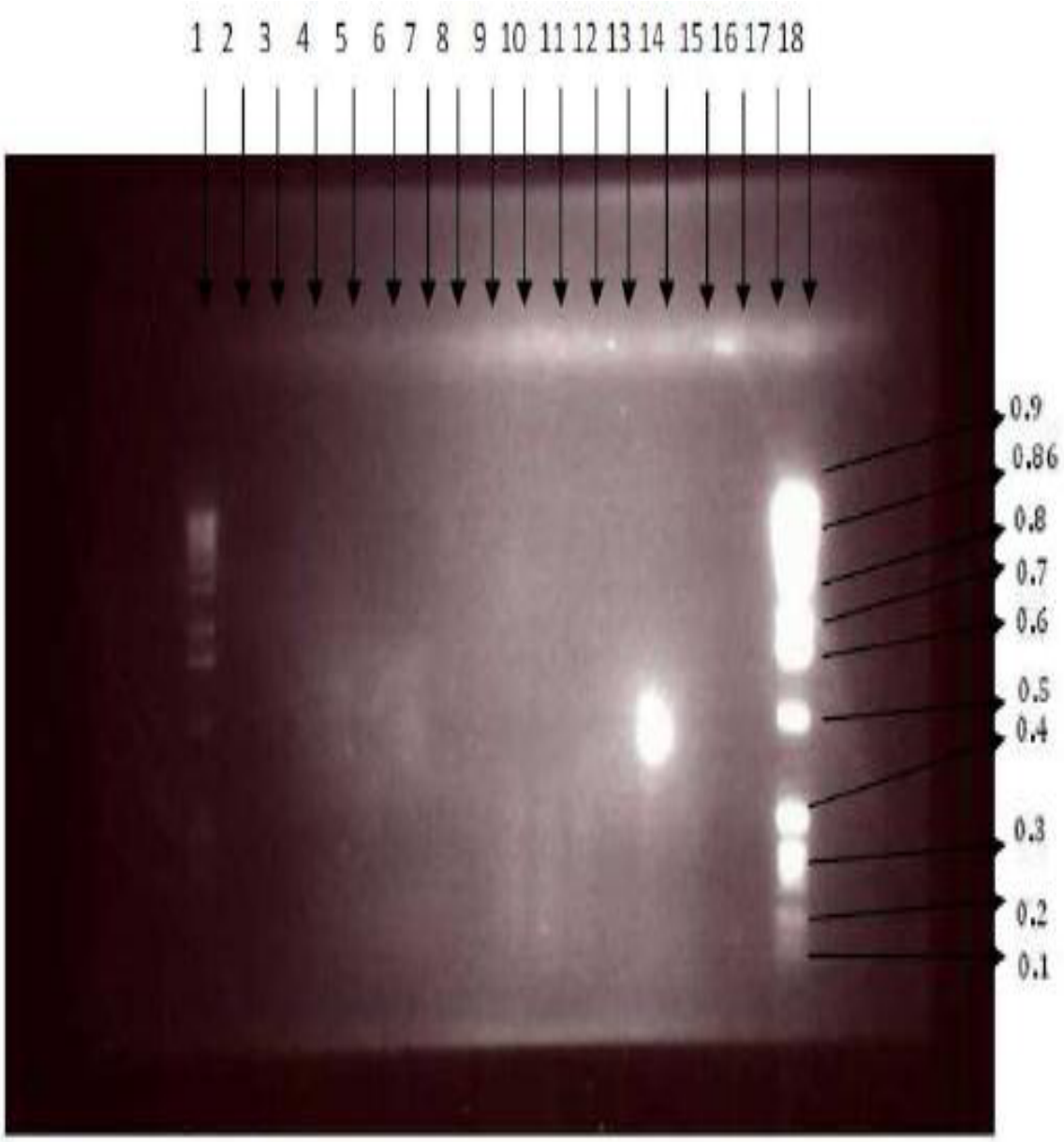

Figure 2 showing the RT-PCR results of HBECs for TLR9 gene that was observed

1, 18 ⟶ Molecular weight markers in kilobase pair;

2, 3, 4, 5, 6, 7 ⟶ TLR 4 samples

10, 11, 12, 13, 14, 15 → TLR 9 samples

8, 9, 16, 17 ⟶ No templates

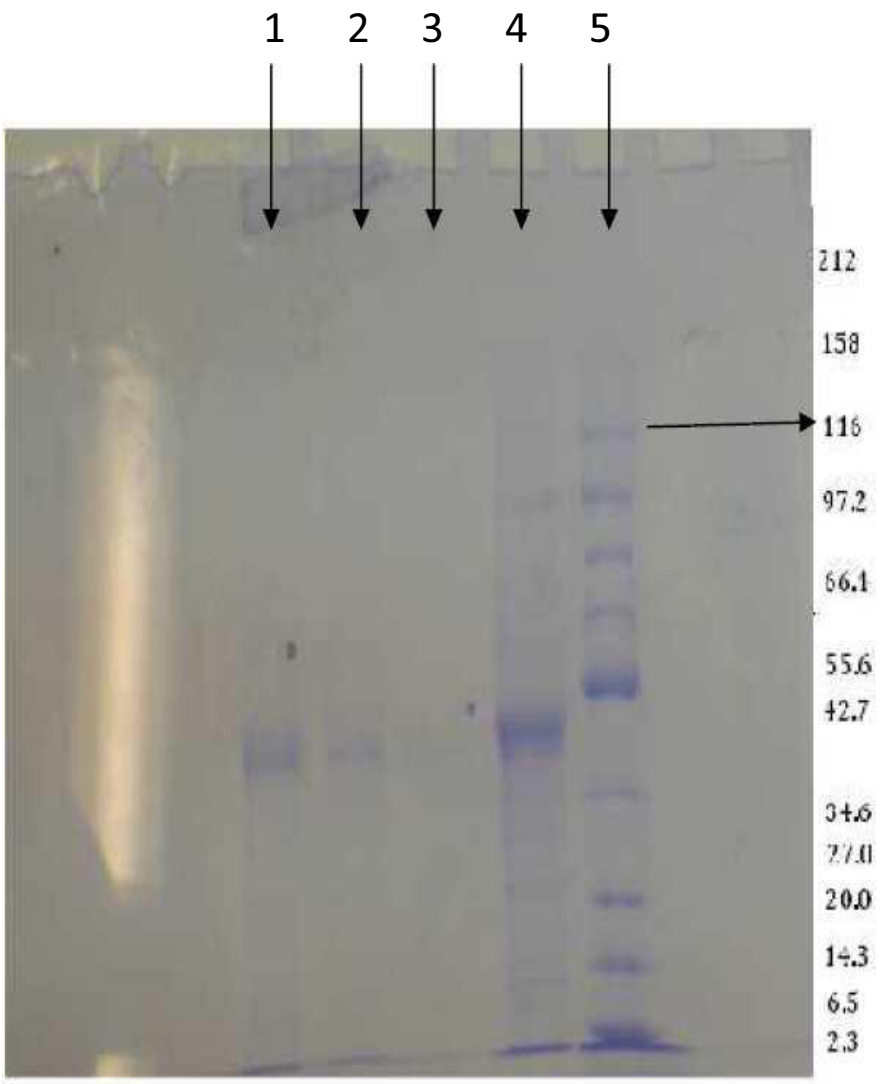

Figure 3 showing the band of HBECs for TLR9 protein in the coomassie blue staining

Lane 1,2,3,4 represents the different concentrations of protein samples and lane 5 represents the protein markers in kDa.

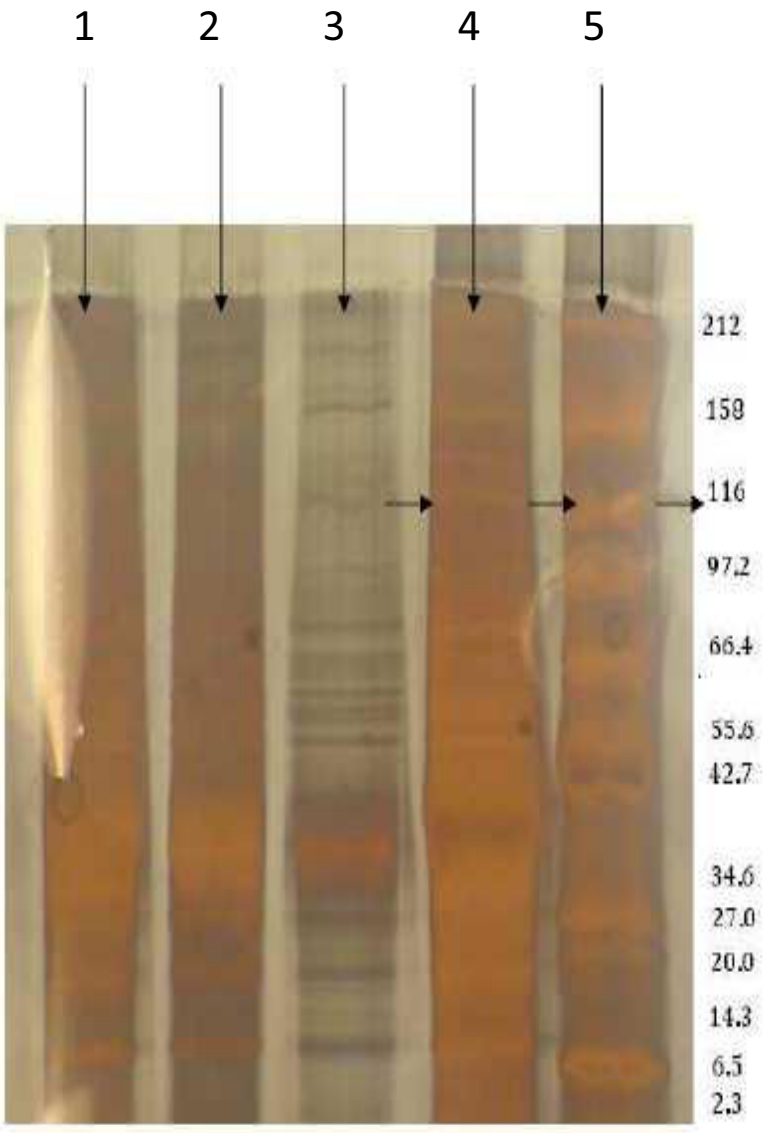

Figure 4 representing the silver staining results of the human bronchial epithelial cells (HBEC) for TLR9 protein.

Lane 1,2,3,4 different concentration of protein samples and lane 5 represents the protein marker in kDa.

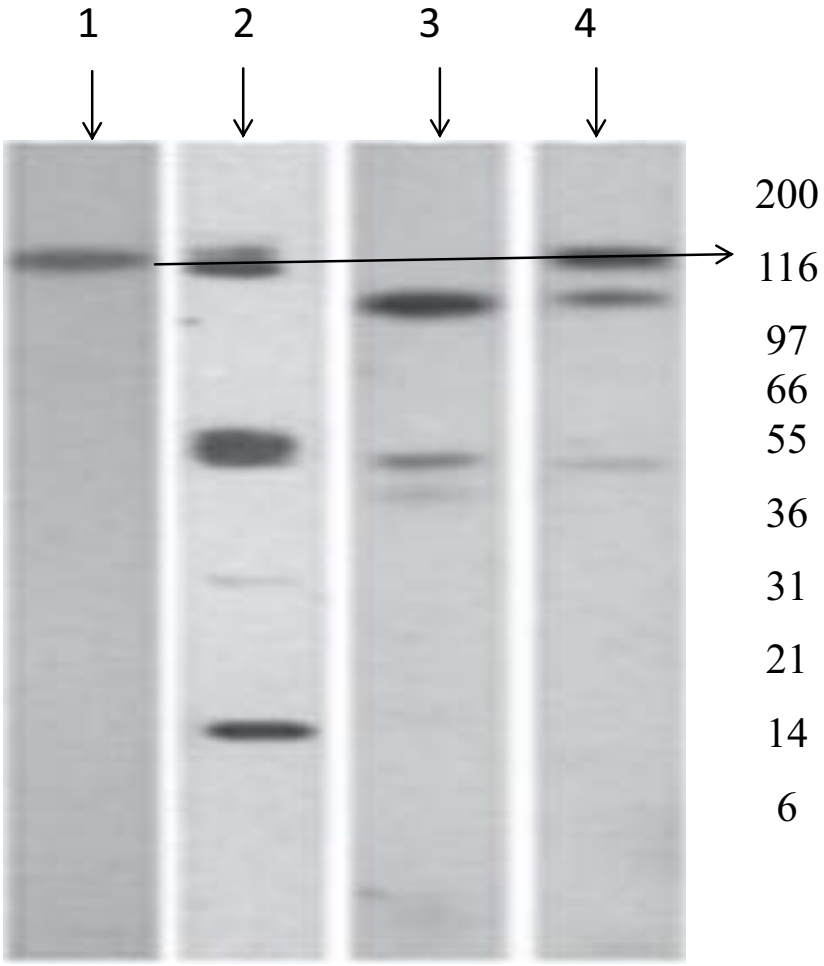

Figure 5 representing the Western blot results of the HBECs induced with LPS.

Lanes 2, 3, 4 representing the different concentration of the protein samples and lane 1 representing the marker for TLR-9